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Discoidin domain receptors guide axons along longitudinal tracts in *C. elegans*

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ABSTRACT

Discoidin domain receptors are a family of receptor tyrosine kinases activated by collagens. Here we characterize the role of the two discoidin domain receptors, *ddr-1* and *ddr-2*, of the nematode *C. elegans* during nervous system development. *ddr-2* mutant animals exhibit axon guidance defects in major longitudinal tracts most prominently in the ventral nerve cord. *ddr-1* mutants show no significant phenotype on their own but significantly enhance guidance defects of *ddr-2* in double mutants. *ddr-1* and *ddr-2* GFP-reporter constructs are expressed in neurons with axons in all affected nerve tracts. DDR-1 and DDR-2 GFP fusion proteins localize to axons. DDR-2 is required cell-autonomously in the PVPR neuron for the guidance of the PVPR pioneer axon, which establishes the left ventral nerve cord tract and serves as substrate for later outgrowing follower axons. Our results provide the first insight on discoidin domain receptor function in invertebrates and establish a novel role for discoidin domain receptors in axon navigation and axon tract formation.

Introduction

During development neurons send out axons, which integrate a vast number of extracellular cues in order to reach their target cells. These guidance cues are detected by receptors on the growth cone at the tip of an extending axon. Receptor–ligand binding triggers intracellular signaling pathways, which affect actin and microtubule assembly, leading to growth cone stabilization or collapse resulting in directed axon outgrowth (Vitriol and Zheng, 2012). Several receptor tyrosine kinases (RTKs) are involved in axon guidance including Ryk/Derailed, which has been implicated in Wnt-mediated axon repulsion (Callahan et al., 1995; Keeble and Cooper, 2006) and Eph receptors which are of particular importance in topographic mapping (Reber et al., 2007). RTKs are type I transmembrane proteins consisting of an intracellular tyrosine kinase domain, a single-pass transmembrane domain and an extra-cellular ligand-binding domain. Human RTKs are classified into 20 families (Hanks and Hunter, 1995; Manning et al., 2002; Robinson et al., 2000). The small family of discoidin domain receptors consists of two members in mammals, DDR1 and DDR2. Likewise, *C. elegans* has two discoidin domain receptor genes, *ddr-1* and *ddr-2*, while only one gene has been identified in *Drosophila* (Vogel et al., 2006). Phylogenetic analysis (Fig. 1A) suggests that the two discoidin domain receptor genes in mammals and *C. elegans* evolved independently from a single ancestral gene (Vogel et al., 2006). In contrast to other RTKs, which typically bind small soluble proteins, mammalian discoidin domain receptors are activated by collagens. Both receptors interact with fibrillar collagens and require a native triple-helical structure for receptor activation (Shrivastava et al., 1997; Vogel et al., 1997). Discoidin domain receptors also interact with members of the network-forming collagens. Collagen type IV and VIII act as ligands for DDR1 while type X collagen interacts with DDR2 (Hou et al., 2001; Leitinger and Kwan, 2006; Shrivastava et al., 1997; Vogel et al., 1997). Unlike most RTKs, which usually dimerize upon ligand binding, discoidin domain receptors form stable, disulfide-linked dimers in the absence of collagen stimulation (Abdulhussein et al., 2008; Mihai et al., 2009; Noordeen et al., 2006). Dimerization of the extracellular domains is a prerequisite for ligand binding (Leitinger, 2003).

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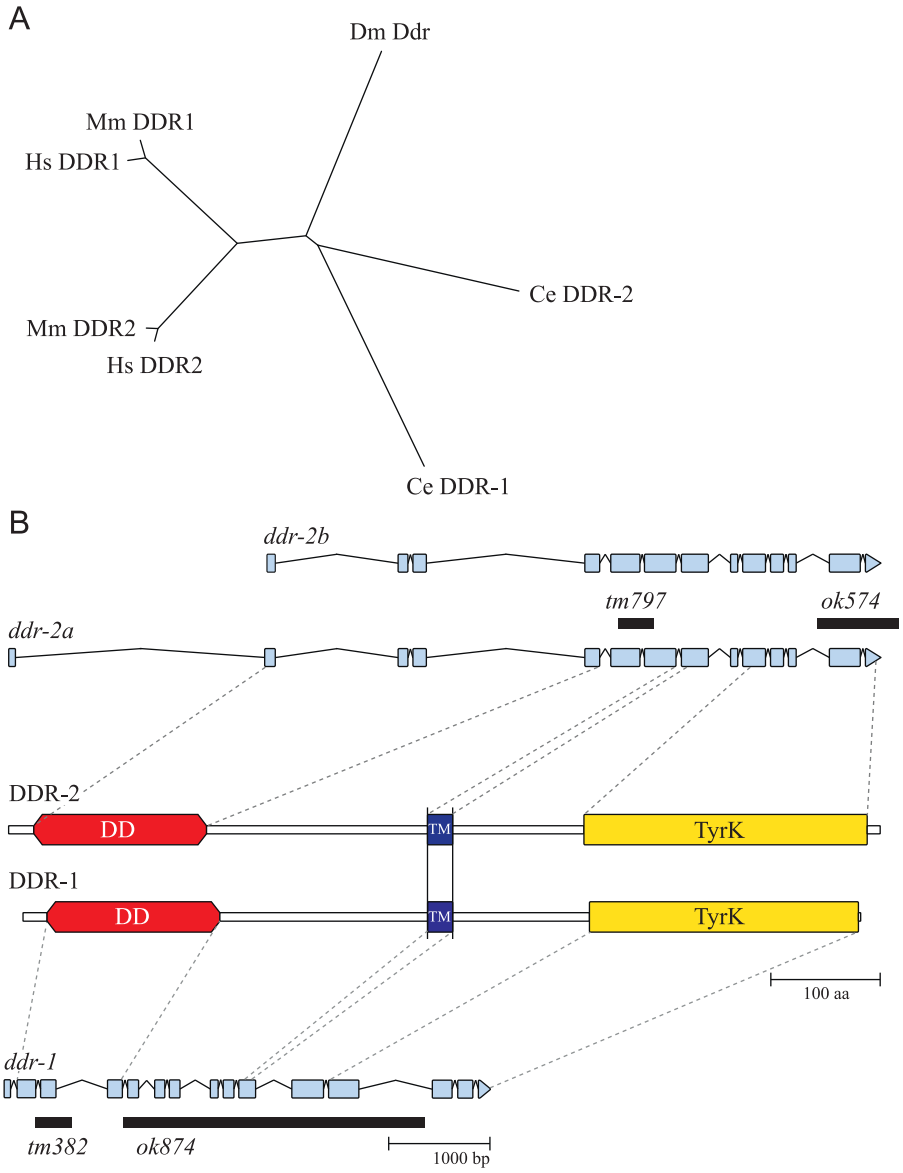

Fig. 1. (A) Relationship between discoidin domain receptors. Unrooted phylogenetic tree of human (Hs DDR), mouse (Mm DDR), *Drosophila* (Dm Ddr) and *C. elegans* (Ce DDR) discoidin domain receptors. (B) Gene models and protein domain organization of discoidin domain receptors in *C. elegans*. The locations of the deletions used in this study are indicated by black bars. DD: discoidin domain; TM: transmembrane domain; TyrK: tyrosine kinase.

Compared to the rapid autophosphorylation of other RTKs, discoidin domain receptor activation is slow and is sustained for a prolonged period of time (Vogel et al., 1997).

Discoidin domain receptors are involved in numerous processes during development such as regulating cell proliferation, adhesion, migration and remodeling of the extracellular matrix in part through the activation of metalloproteinases (Curat and Vogel, 2002; Hou et al., 2001,2002; Labrador et al., 2001; Olaso et al., 2002). Both discoidin domain receptors are expressed in the nervous system. DDR2 expression was found in the developing and mature brain of rats (Lai and Lemke, 1994). DDR1 is expressed in proliferating areas in the central nervous system of rats and mice (Sanchez et al., 1994; Zerlin et al., 1993). Postnatally, DDR1 expression follows the progress of myelination and was detected in both myelin and oligodendrocytes (Franco-Pons et al., 2006; Roig et al., 2010). In humans DDR1 has been suggested as a susceptibility gene for schizophrenia (Roig et al., 2007).

In this study we characterize discoidin domain receptors in the nematode *C. elegans*. We found that *ddr-1* and *ddr-2* are expressed in the nervous system *ddr-2* mutants show a variety of axon navigation defects in major longitudinal tracts, most notably the left ventral nerve cord. DDR-2 is required cell-autonomously in the left ventral nerve cord pioneer neuron PVPR for the proper guidance of both the pioneer and follower axons. Mutants in *ddr-1* show no phenotype alone but act synergistically with *ddr-2* highlighting a novel role for the two discoidin domain receptors in axon guidance during nervous system development of *C. elegans*.

Results

Discoidin domain receptors in C. elegans

Discoidin domain receptors are single-pass transmembrane proteins. Their extracellular region contains the ligand-binding discoidin domain and a stalk region. The intracellular region consists of a juxtamembrane region and the catalytic tyrosine kinase domain (Fig. 1B). The *ddr-1* gene of *C. elegans* encodes a

protein with 766 amino acids. *ddr-2* has two alternative splice forms with the product of *ddr-2a* comprising 797 amino acids and 767 amino acids for isoform *ddr-2b* (Fig. 1B) (Gerstein et al., 2010; Yook et al., 2012).

Deletion alleles for *ddr-1* and *ddr-2* are available from the *C. elegans* Knockout Consortium and the National BioResource Project (Moerman and Barstead, 2008). The 357 bp deletion *ddr-1*(*tm382*) results in an early stop codon truncating the DDR-1 protein after amino acid 94 in the discoidin domain. *ddr-1*(*ok874*) is a large 2975 bp deletion which eliminates most of the *ddr-1* gene and causes a frame shift resulting in an early stop after 179 amino acids in the extracellular domain. Both *ddr-1* alleles remove the tyrosine kinase and transmembrane domains and thus likely result in loss-of-function (Fig. 1B). *ddr-2*(*ok574*) is a 809 bp deletion and 17 bp insertion which removes the last two exons of the DDR-2 protein containing part of the kinase domain (Fig. 1B). *ddr-2*(*tm797*) is a 349 bp deletion resulting in a premature stop after amino acid 234 in the extracellular domain. Both alleles remove or

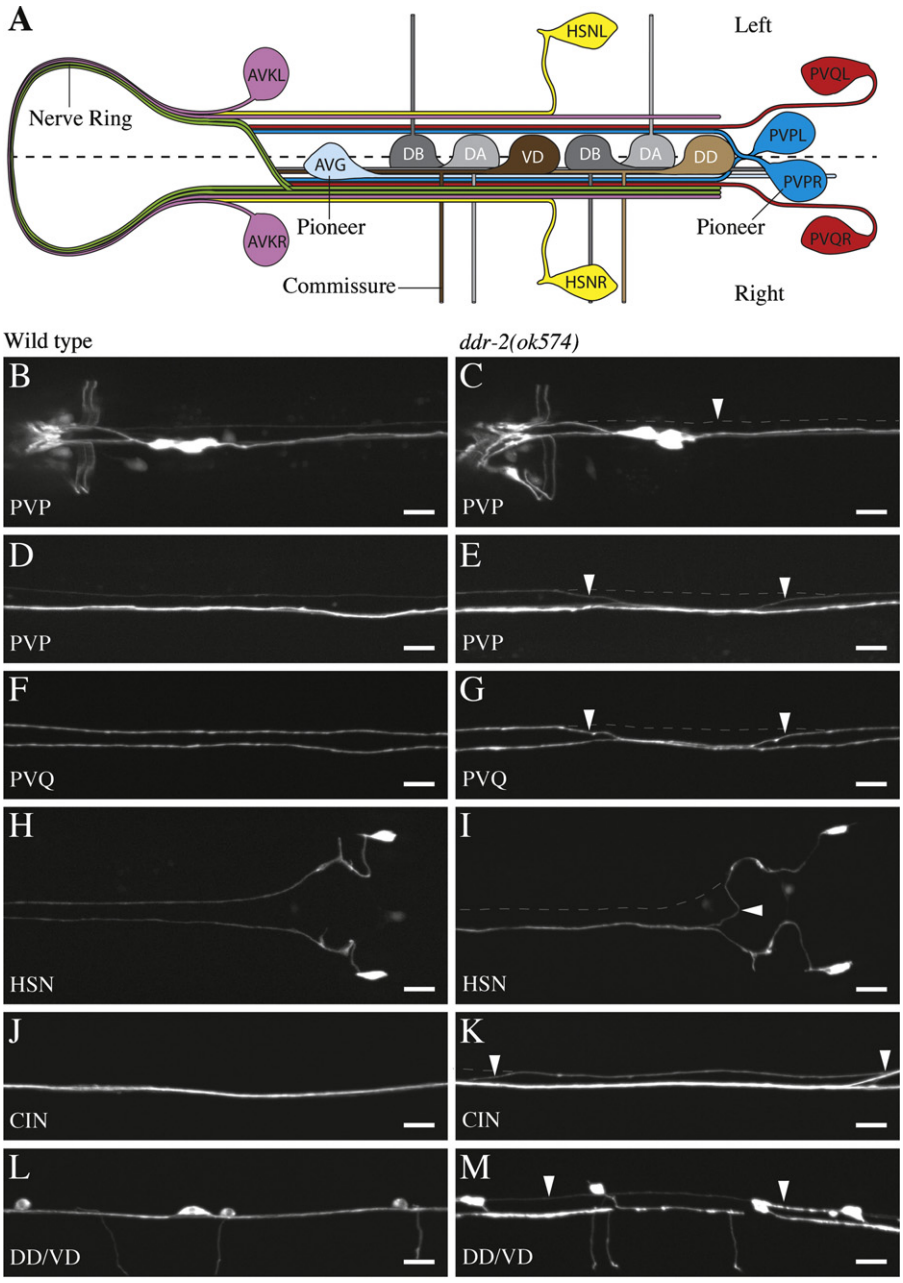

Fig. 2. (A) Ventral nerve cord (VNC) of *C. elegans*. The two VNC tracts are separated by motor neuron cell bodies, the right VNC tract is pioneered by the axon of AVG, the left tract by the axon of PVPR. Note that PVP and AVK axons cross the ventral midline, so that the PVPR and AVKR axons end up in the left axon tract, whereas PVPL and AVKL are in the right axon tract. All axons with the exception of the post-embryonic HSN axons grow out in the embryo. (B–M) Axon trajectories in wild type (B, D, F, H, J, L) and *ddr-2*(*ok574*) mutants (C, E, G, I, K, M) visualized by fluorescent reporter constructs. (B, D) In wild type, the PVPR axon is in the left VNC tract. (C) In *ddr-2* mutant animals, PVPR fails to pioneer the left tract (arrowhead) and instead grows in the right tract or (E) switches between the two tracts (arrowheads). (G) The PVQL axon follows the misled PVPR pioneer (arrowheads). (H) In wild type, the HSNL axon extends in the left VNC tract. (I) In *ddr-2* mutant animals, the axon of HSNL joins the right VNC tract (arrowhead). (J) In wild type, command interneuron axons extend in the right VNC tract. (K) In *ddr-2* mutant animals, axons switch into the left VNC tract (arrowheads). (L) In wild type, the longitudinal processes of DD/VD motor neurons extend into the right VNC tract. (M) In *ddr-2* mutant animals, some DD/VD processes join the left tract (arrowheads). Dashed lines in pictures reflect normal axon trajectories. All pictures and the diagram show ventral views; anterior is to the left. Fluorescent reporter constructs used: AVG, PVPR/L: *odr-2*::*CFP*; PVQR/L: *sra-6*::*DsRed2*; HSNR/L: *tph-1*::*GFP*; AVKR/L: *flp-1*::*GFP*; command interneurons (CIN): *glr-1*::*GFP*; DD/VD motor neurons: *unc-47*::*DsRed2*. Scale bar: 10 μm.

disrupt the intracellular kinase domain and therefore likely represent strong loss-of-function or even molecular null alleles of *ddr-2*. All *ddr-1* and *ddr-2* single mutants or double mutant combinations of *ddr-1 and ddr-2* analyzed in this study were viable and showed no obvious anatomical defects or movement impairment.

ddr-2 mutants show axon guidance defects in major longitudinal tracts, which are enhanced in ddr-1 ddr-2 double mutants

Expression data from different embryonic tissues indicate that *ddr-1* and *ddr-2* are highly expressed in embryonic neurons (McKay et al., 2003), raising the possibility that these genes might have a role in nervous system development. We analyzed the nervous system of both *ddr-1* and *ddr-2* mutants with various cell-type specific markers and found axon guidance defects in several longitudinal axon tracts in *ddr-2* mutant animals. *C. elegans* has two major longitudinal fascicles, the ventral nerve cord (VNC) and the dorsal nerve cord (DNC). The VNC consists of two nerve bundles separated by motor neuron cell bodies arranged in a line at the ventral midline (Durbin, 1987; White et al., 1976,1986). Most of the axons extend in the right fascicle; only four axons run in the left tract (Fig. 2A). Each VNC tract is initially established by a single axon (Durbin, 1987). The process of the AVG neuron, located at the anterior end of the VNC, pioneers the right tract. The left tract is pioneered by the axon of the PVPR neuron, which extends anteriorly from the posterior end of the VNC (Fig. 2A). Whereas *ddr-2* mutants had only very mild defects in the AVG pioneer, in more than one third of *ddr-2*

Table 1
Axon guidance defects in ventral nerve cord (% animals with defects).

Allele	VNC asym.[a]	Right tract							Left tract			
		AVG	PVPL	PVQR	AVKL	HSNR	CIN	DD/VD	PVPR	PVQL	AVKR	HSNL
Wild type	0	0	0 (0)	0 (0)	2	3	2	8	11 (0)	13 (0)	7	9
ddr-2(*ok574*)	5**	7**	7** (0)	7** (1)	11**	6	9**	28**	38** (10**)	37** (9**)	13	27**
ddr-2(*tm797*)	2*	5**	5** (0)	8** (1)	7	10	7*	29**	44** (11**)	44** (11**)	4	30**
ddr-1(*ok874*)	0	0	0 (0)	0 (0)	2	6	6	7	15 (4*)	16 (3*)	5	5
ddr-1(*tm382*)	2	0	1 (1)	0 (0)	n.d.	n.d.	6	n.d.	19 (2)	22 (2)	n.d.	n.d.
ddr-2(*ok574*) *ddr-1*(*ok874*)	6	3	9 (0)	8 (1)	6	9	16	41$	61$$ (22$)	61$$ (22$)	13	47$$
ddr-2(*tm797*) *ddr-1*(*tm382*)	3	8	13$$ (1)	16$ (1)	n.d.	n.d.	13	n.d.	65$$ (23$$)	62$$ (24$$)	n.d.	n.d.

$n \geq 100$ for each data point, markers used: *VNC asym.*: *rgef-1*::*GFP*; AVG, PVPR/L: *odr-2*::*CFP*; PVQR/L: *sra-6*::*DsRed2*; AVKR/L: *flp-1*::*GFP*; HSNR/L: *tph-1*::*GFP*; command interneurons (CIN): *glr-1*::*GFP*; DD/VD motor neurons*: unc-47*::*DsRed2*, numbers in brackets reflect % of animals with axon extending over the entire distance in the contralateral axon tract. n.d.: not determined, $/$$: penetrance of defects in double mutants compared to defects observed for strongest single mutant. */$ $P < 0.05$ (χ^2 test), **/$$ $P < 0.01$ (χ^2 test).

[a] Ventral nerve cord asymmetry defect: axons fail to cross into right VNC tract after leaving nerve ring.

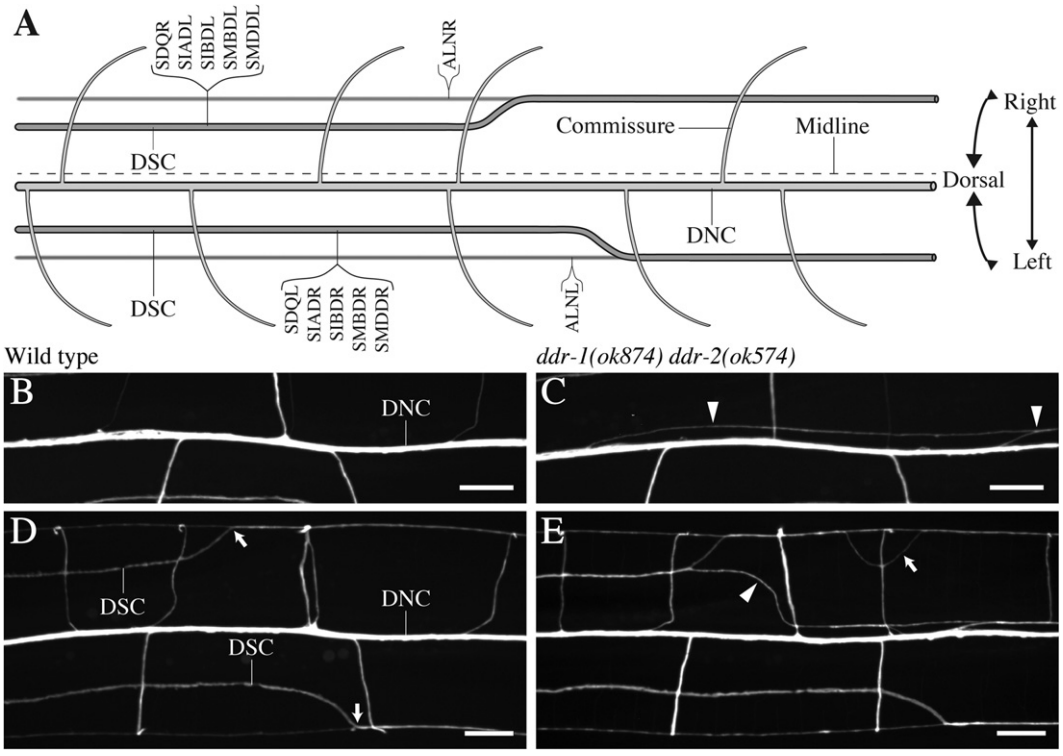

Fig. 3. (A) Dorsal nerve cord and dorsal sublateral cords. Commissure axons from ventral motor neurons migrate circumferentially along both sides of the body towards dorsal midline and form the dorsal nerve cord (DNC) on the left side of the midline. The two dorsal sublateral cords (DSC) flank the dorsal nerve cord on either side. (B–E) Wild type and *ddr-1*(*ok874*) *ddr-2*(*ok574*) double mutant animals labeled with pan-neuronal GFP. (B) In wild type animals, the dorsal nerve cord is tightly fasciculated, all axons extend on the left side of the dorsal midline. (C) In *ddr-1 ddr-2* double mutants, some axons extend on the right side of the midline (arrowheads). (D) Axons from the dorsal sublateral tracts join the ALN axon trajectories (arrows). (E) In *ddr-1 ddr-2* double mutants, axons from the right dorsal sublateral cord grow dorsally and extend along the right side of the dorsal midline (arrowhead); other axons leave their normal trajectory (arrow). All pictures and the diagram show dorsal views; anterior is to the left. Fluorescent marker used: *rgef-1*::*GFP*. Scale bar: 10 μm.

mutant animals the axon of the PVPR pioneer neuron crossed the ventral midline to join the contralateral tract or failed to establish the left axon tract altogether (Fig. 2C, E; Table 1).

The PVPR pioneer axon in the left tract is closely followed by the PVQL axon (Durbin, 1987). PVPR and PVQL in the left tract are subsequently joined by the AVKR axon, growing posteriorly from the nerve ring; and post-embryonically by the axon of HSNL, which joins the VNC tract in the midbody region and extends anteriorly (Fig. 2A).

In *ddr-2* mutant animals the later outgrowing axons from PVQL and HSNL neurons followed the misled pioneer, suggesting that defects in these neurons are a secondary consequence of a primary defect in PVPR (Fig. 2G, I; Table 1). The AVKR axon was not affected.

The situation in the right VNC tract is more complex due to the larger number of axons in this fascicle. Similar to the left tract, axon outgrowth follows a distinct pattern with the pioneer and various follower axons growing out sequentially. In *ddr-2* mutant animals PVPL and PVQR follower axons were weakly affected while HSNR axon navigation was not significantly disturbed (Table 1). Command interneuron axons descending from the nerve ring normally extend in the right VNC tract. In *ddr-2* mutant animals command interneurons occasionally crossed into the left VNC tract (Fig. 2K; Table 1). DD/VD and DA/DB motor neurons each extend one process in the right VNC axon tract. In addition, they extend commissures circumferentially towards the dorsal midline to form the dorsal nerve cord. In nearly one third of the *ddr-2* mutant animals DD/VD motor neuron axons aberrantly joined the left VNC tract (Fig. 2M; Table 1). DD/VD commissure navigation was not significantly disturbed. DA/DB axons were generally unaffected in *ddr-2* mutants (data not shown).

We did not observe any substantial axon guidance defects in the VNC of *ddr-1* mutant animals (Table 1). However, *ddr-1 ddr-2* double mutants exhibited strongly increased defects in the PVPR pioneer (Table 1), suggesting that the two discoidin domain receptors act synergistically in guiding the left VNC pioneer. With few exceptions, later outgrowing axons from PVQL and HSNL followed the misguided pioneer. As in each single mutant, AVKR axons were undisturbed in *ddr-1 ddr-2* double mutants. The penetrance of axon guidance defects in the right VNC tract was slightly enhanced in PVPL and PVQR and DD/VD motor neurons, which showed an increase of axons extending in the left VNC tract (Table 1).

In addition to defects in the VNC we also observed defects in other longitudinal axon tracts. The dorsal nerve cord (DNC) runs longitudinally on the left side of the dorsal midline. It is formed by motor neuron axons that branch and extend in anterior and posterior directions after growing circumferentially on both sides of the body from the VNC (Fig. 3A). We found that in a quarter of *ddr-2(tm797)* mutant animals and in nearly half of the *ddr-1 ddr-2* double mutant animals some axons extended on the right side of the dorsal midline (Fig. 3C, Table 2). In these animals motor neuron axons, which grew circumferentially along the right side of the body, branched on the right side of the dorsal midline and extended longitudinally before crossing the dorsal midline to join the DNC. However, we cannot exclude that a subset of the axons extending on the right side of the dorsal midline might have originated from commissures growing circumferentially along the left side of the body, which failed to stay in the DNC and instead crossed the dorsal midline and extended on the right side of the dorsal midline.

The DNC is flanked by two dorsal sublateral cords (DSCs), which run in parallel to the DNC until they turn ventrally to join the ALN axon in the midbody region (Fig. 3A, D). In a significant number of *ddr-2* mutant animals; and even more often in *ddr-1 ddr-2* double mutant animals, axons from the right DSC turned dorsally and grew along the right side of the dorsal midline (Fig. 3E, Table 2). Axons from the left DSC were less frequently affected (Table 2). Also, some DSC axons left their normal trajectory (Fig. 3E) or converged with the ALN axon at a more anterior or posterior position (Table 2). Similar to the DNC, the VNC is flanked by two ventral sublateral cords (VSCs). The right VSC was mildly affected by mutations in *ddr-2*, loss of *ddr-1* had no effect on either VSC (Table 2).

Since discoidin domain receptors have been described to affect the migration of mammalian cells (Hou et al., 2001,2002; Kamohara et al., 2001; Ram et al., 2006), we examined whether *ddr-1* and *ddr-2* are important for neuronal cell migrations in *C. elegans*. We did not observe significant migration defects in *ddr-1* and *ddr-2* single mutants or *ddr-1 ddr-2* double mutants for any of the neurons tested (BDU, SDQ, AVM, ALM, HSN, PVM; data not shown). None of the markers used to evaluate axon trajectories or neuronal cell body positions were misexpressed, suggesting that cell fate decisions are probably unaffected in the tested neurons.

Our results show that *ddr-1* and *ddr-2* are involved in axon guidance in the major longitudinal axon tracts of *C. elegans*. While

Table 2
Defects in dorsal nerve cord and sublateral cords (% animals with defects).

Allele	DNC[a]	Right DSC[b]		Left DSC[b]		Right VSC[c]		Left VSC[c]	
		Nav.[d]	Total[e]	Nav.[d]	Total[e]	Nav.[f]	Total[g]	Nav.[f]	Total[g]
Wild type	7	0	2	0	1	0	1	0	4
ddr-2(*ok574*)	14	4*	6	0	1	2	5	0	2
ddr-2(*tm797*)	24**	4*	11**	1	13**	4*	8*	0	6
ddr-1(*ok874*)	12	2	7*	2	7*	0	1	0	1
ddr-1(*tm382*)	19*	3*	7*	3*	5	0	4	0	2
ddr-2(*ok574*) *ddr-1*(*ok874*)	44$$	19$$	23$$	5	15$	4	11	0	6
ddr-2(*tm797*) *ddr-1*(*tm382*)	47$$	23$$	34$$	10$	21	4	12	0	7

$n \geq 100$ for each data point, marker used: *rgef-1*::*GFP* (pan-neuronal), $/$$: penetrance of defects in double mutants compared to defects observed for strongest single mutant. */$ $P < 0.05$ (χ^2 test), **/$$ $P < 0.01$ (χ^2 test).

[a] Dorsal nerve cord midline crossing defect and/or DSC navigation defect ([d]) (see Fig. 3C, E).

[b] Dorsal sublateral cord.

[c] Ventral sublateral cord.

[d] Navigation defects: axons leave DSC, extend dorsally towards DNC and grow posteriorly next to dorsal midline (see Fig. 3E).

[e] Total defects, including animals with axons leaving DSC, axons with navigation defects ([d]) and animals with axons converging with ALN axon trajectory at more anterior or posterior position.

[f] Navigation defects: axons leave VSC and grow ventrally to join the ventral nerve cord.

[g] Total defects, including animals with axons leaving VSC, axons with navigation defects ([f]) and animals with position of VSC axons ascending dorsally shifted anteriorly or posteriorly.

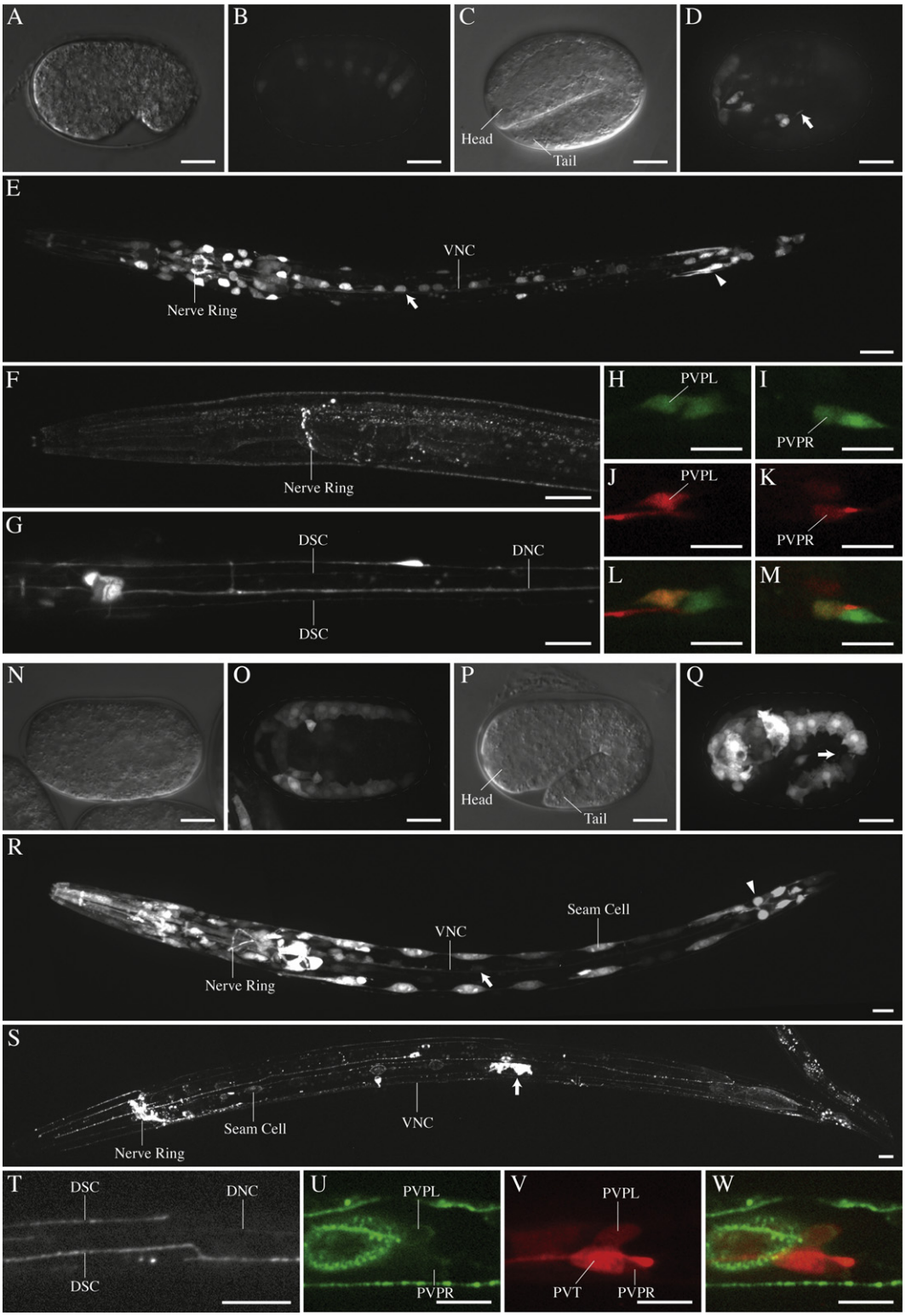

Fig. 4. Discoidin domain receptor expression and localization. (A, B) Onset of *ddr-1p*::*GFP* expression in the embryo during "lima-bean-stage". (C, D) 2-fold-stage embryo showing an extending axon (arrow). (E) L1 larva, *ddr-1p*::*GFP* is expressed in neurons in head and tail ganglia, motor neurons (arrow) and stomato-intestinal muscle (arrowhead). (F) Adult animal, DDR-1::GFP fusion protein localizing to axons in the nerve ring. (G) L1 larva, *ddr-1p*::*GFP* is expressed in dorsal nerve cord (DNC) and dorsal sublateral tracts (DSC). (H–M) Coexpression of *ddr-1p*::*GFP* (green) with *odr-2*::*CFP* (red) in PVP neurons.
(N, O) *ddr-2p*::*GFP* expression starts during late gastrulation. (P, Q) 1.5-fold-stage embryo with an extending axon (arrow). (R) L1 larvae, *ddr-2p*::*GFP* is expressed in neurons in head and tail, motor neurons (arrow), rectal gland cells (arrowhead) and seam cells. (S, T) L2 larvae; DDR-2::GFP signal localizes to the nerve ring and all major longitudinal axon tracts, seam cells and most likely uterine valve cells of the vulva (arrow). (U–W) Coexpression of DDR-2::GFP (green) with *odr-2*::*CFP* (red) in PVP neurons. (A–D, F, N–Q, S) Lateral views; (T) dorso-lateral view; (E, H–M, R, U–W) ventral views; (G) dorsal view. In all pictures anterior is to the left. (G, H–M, T) single focal plain. (A–G, N–T) Scale bar: 10 μm; (H–M, U–W) scale bar: 5 μm. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article).

mutations in *ddr-1* did not result in defects on their own, we found that axon guidance defects in *ddr-1 ddr-2* double mutants were significantly enhanced compared to *ddr-2* single mutants, indicating that the two discoidin domain receptors act together in axon guidance in *C. elegans*.

ddr-1 and ddr-2 are expressed in the nervous system and non-neuronal tissues

We created transgenic strains expressing transcriptional and translational GFP reporter constructs to visualize the expression pattern and subcellular localization of *ddr-1* and *ddr-2* during development. Expression of a GFP reporter gene under the control of the *ddr-1* promoter (*ddr-1p*::*GFP*) was first detected in the "lima-bean-stage" during embryogenesis in hypodermal cells (Fig. 4B). During the early stages of axon outgrowth in the embryo, around the 2-fold stage, GFP expression included a few head and tail neurons (Fig. 4D). Post-embryonically, *ddr-1p*::*GFP* expression was mostly observed in the nervous system, with many neurons in head and tail ganglia and motor neurons in the VNC expressing GFP (Fig. 4E). Expression of *ddr-1p*::*GFP* in PVP neurons was confirmed by co-labeling with a PVP marker (Fig. 4H–M). Outside the nervous system expression was apparent in the pharynx and the stomato-intestinal muscle (Fig. 4E).

ddr-2p::*GFP* reporter gene expression began at the end of gastrulation (Fig. 4O) with strong expression in seam cells and later also in a few neurons in the head (Fig. 4Q). Post-embryonically neuronal expression was limited to a subgroup of neurons in the head and tail and some motor neurons in the VNC (Fig. 4R). Expression outside the nervous system was most prominent in seam cells, rectal gland cells and several non-neuronal cells in the tail (Fig. 4R).

To determine the subcellular localization of DDR-1 and DDR-2, we generated constructs, with GFP fused to the C-terminus of each protein. These translational fusion constructs, DDR-1::GFP and DDR-2::GFP, localized to neuronal cell bodies and axons (Fig. 4F, S, T). DDR-2::GFP co-localized with a PVP marker confirming the expression of DDR-2 in the PVP neurons (Fig. 4U–W).

Both discoidin domain receptors showed neuronal expression during the time of axon outgrowth in the embryo (Fig. 4D, Q). In L1 larvae *ddr-1* and *ddr-2* GFP-reporter constructs expressed in neurons with axons in all four sublateral cords and the ventral and dorsal nerve cord (Fig. 4E, G, R, S, T), which is consistent with the idea that *ddr-1* and *ddr-2* function in the affected neurons.

DDR-2 functions cell-autonomously in the PVPR pioneer neuron

We performed a genetic mosaic analysis to test whether *ddr-2* acts cell-autonomously in the PVPR neuron by correlating the presence or absence of a rescuing *ddr-2* transgene in PVPR with the presence or absence of axonal defects (see material and methods for details). We found that loss of an extra-chromosomal array containing a functional *ddr-2* gene in PVPR abolished the rescue of the pioneer navigation defects (Fig. 5A). Similarly loss of the transgene in PVPL abolished the rescue of PVPL defects (Fig. 5A), suggesting that both PVP neurons require *ddr-2* cell-autonomously. By contrast, loss of the *ddr-2* transgene in the PVQ neurons did not lead to enhanced PVQ navigation defects (Fig. 5A), consistent with the idea that the PVQ follower defects are a consequence of the PVP pioneer defects, i.e. PVQ axons follow the misguided pioneer axons. This was further confirmed by testing explicitly, whether the transgene was required in PVPR or PVQL to rescue the PVQL defects (Fig. 5B). Loss of the *ddr-2* transgene in PVPR, but not in PVQL, lead to PVQL axon navigation defects comparable to those in *ddr-1 ddr-2* double mutants (Fig. 5B). Taken together these results indicate that *ddr-2* functions cell-autonomously in the PVPR neuron to guide the pioneering axon and that the PVQL follower defects are a secondary consequence of pioneer defects.

ddr-1 and ddr-2 interact genetically with nid-1/nidogen and fmi-1/flamingo

To identify potential interaction partners for *ddr-1* and *ddr-2* we created double mutants and triple mutants with potential guidance cues and receptors that have been shown to affect PVPR axon navigation and collagens, which have been identified as

A

		Transgene	% Defects	n	Transgene	% Defects	n
Right VNC	AVG	+	3	378	-	6	17
	PVPL	+	3	553	-	**26****	47
	PVQR	+	5	560	-	3	31
Left VNC	PVPR	+	21	561	-	**61****	38
	PVQL	+	25	553	-	21	33

B

Presence of Transgene					% PVQL Defects	n
Right VNC			Left VNC			
AVG	PVPL	PVQR	PVPR	PVQL		
+	+	+	+	+	24	459
-	+	+	+	+	10	10
+	-	+	+	+	25	24
+	+	-	+	+	19	21
+	+	+	-	+	**52****	23
+	+	+	+	-	17	24
-	-	-	-	-	67	88

Fig. 5. Mosaic analysis. (A, B) Presence (+) or absence (–) of the rescuing extrachromosomal array containing *ddr-2*(+) in AVG, PVP and PVQ neurons was determined by the presence of GFP reporter gene expression. (A) Percentages reflect axon guidance defects in the corresponding neuron. (B) Percentages show axon guidance defects in PVQL neurons. $^{**}P < 0.01$ (χ^2 test). Fluorescent markers used: AVG, PVPR/L: *odr-2*::*CFP*; PVQR/L: *sra-6*::*DsRed2*. Co-injection markers for array identification: *odr-2*::*GFP*; *sra-6*::*GFP*.

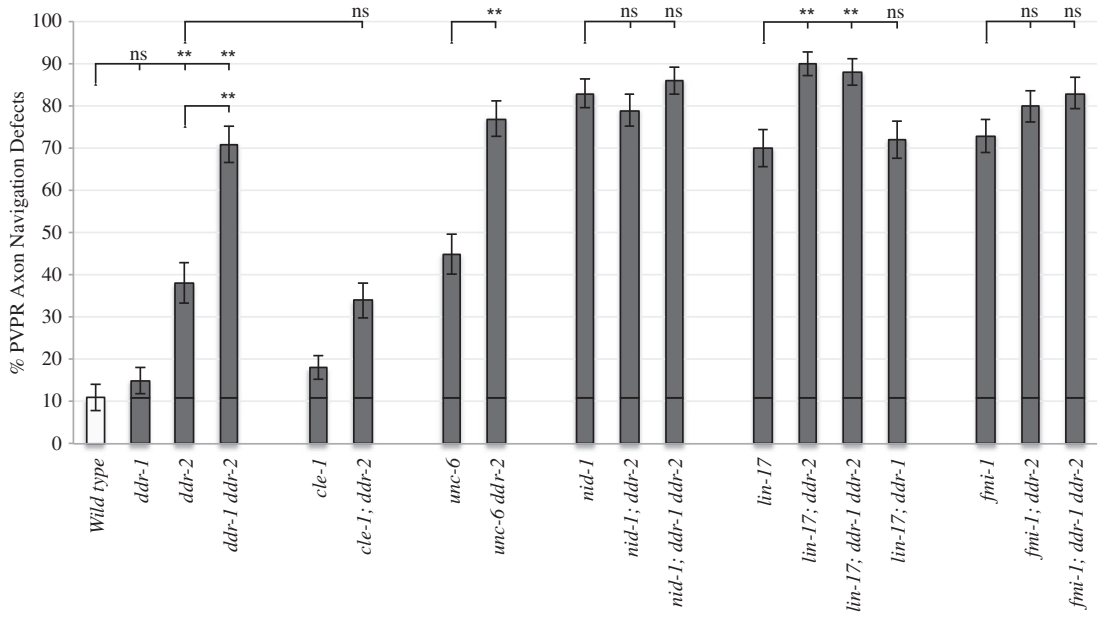

Fig. 6. Genetic interactions between discoidin domain receptors and selected axon guidance genes. Columns reflect percentages of animals with PVPR pioneer navigation defects ± standard error. For each strain ≥ 100 animals were analyzed. The χ^2 test was used to determine whether the double (or triple mutant) is significantly different from the strongest single mutant (**: $P < 0.01$; ns: not significant). Mutant alleles used: ddr-1(ok874); *ddr-2(ok574); cle-1(cg120); nid-1(cg119); unc-6(ev400); lin-17(n671); fmi-1(rh308)*. Fluorescent marker used: *odr-2::CFP*.

ligands for discoidin domain receptors in mammals (Shrivastava et al., 1997; Vogel et al., 1997). The only collagen known to be involved in axon guidance in *C. elegans* is CLE-1, the homolog of the basement membrane collagens XV/XVIII. Mutations in *cle-1* cause mild defects in motor neuron commissures (Ackley et al., 2001). While no VNC defects have been described to date in *cle-1* mutants, CLE-1 protein accumulates strongly on nerve tracts, including the VNC (Ackley et al., 2001). We found no significant axon guidance defects in the PVPR pioneer neuron (Fig. 6) or in any of the other VNC neurons tested in *cle-1* mutant animals (data not shown). *cle-1*; *ddr-2* double mutants showed no enhanced phenotype compared to the *ddr-2* single mutant (Fig. 6) suggesting that *cle-1* and *ddr-2* do not interact synergistically.

The basement membrane component NID-1/Nidogen affects the navigation of PVPR and PVQL (Hutter, 2003; Kim and Wadsworth, 2000). We found that a majority of the *nid-1* mutant animals had defects in PVPR axon navigation confirming previous results (Fig. 6). PVPR defects were not enhanced in either *ddr-2*; *nid-1* double or *ddr-1 ddr-2*; *nid-1* triple mutants compared to *nid-1*, the strongest single mutant. Taken together, our results indicate that the discoidin domain receptors act in the same genetic pathway as *nid-1*/*nidogen* with respect to PVPR axon guidance.

One of the guidance cues involved in PVPR pioneer guidance is UNC-6/Netrin (Wadsworth et al., 1996). Similar to *ddr-2* mutants, *unc-6* null mutants exhibited partially penetrant guidance defects in the PVPR pioneer axon (Fig. 6). Defects in *ddr-2 unc-6* double mutants were significantly enhanced compared to the single mutants, suggesting that *ddr-2* acts in parallel to the *unc-6*/*netrin* pathway.

Recently, the Wnt receptor LIN-17/Frizzled and the non-classical cadherin FMI-1/Flamingo have been implicated in PVPR pioneer and PVQL follower navigation (Steimel et al., 2010). We observed that PVPR defects in *lin-17*; *ddr-2* double mutants were significantly stronger than in each single mutant indicating that *ddr-2* functions independently of *lin-17*/*frizzled* (Fig. 6). By contrast, PVPR defects were not increased in either *ddr-2*; *fmi-1* double or *ddr-1 ddr-2*; *fmi-1* triple mutants compared to the strongest single mutant (Fig. 6), suggesting that the two discoidin domain receptors and *fmi-1*/*flamingo* function in the same genetic pathway with respect to PVPR pioneer navigation.

Discussion

Discoidin domain receptors ddr-1 and ddr-2 act synergistically to guide axons along longitudinal tracts

In this study we examined the role of discoidin domain receptors during nervous system development in *C. elegans*. In mammals, both DDR1 and DDR2 are expressed in the developing nervous system (Lai and Lemke, 1994; Sanchez et al., 1994; Zerlin et al., 1993). DDR1 is required for axon extension of granule neurons (Bhatt et al., 2000). A dominant-negative form of DDR1 reduced axon extension in granule cell cultures and cerebellar organ cultures. Granule cell axon extension is likely induced by receptor–ligand interaction between DDR1 on granule cells and collagen on the pial layer of the developing cerebellum (Bhatt et al., 2000). In contrast to the observations for DDR1 in the cerebellum of mammals, we found no evidence for axonal outgrowth or extension defects in *C. elegans ddr-1* and *ddr-2* loss-of-function mutants for any of the neurons analyzed. However, *ddr-2* mutants showed significant axon navigation defects in the ventral nerve cord, dorsal nerve cord and dorsal sublateral tracts. Individual axons or whole axon fascicles failed to navigate along their normal trajectories and instead joined other axon tracts in their vicinity. *ddr-1* mutant animals had no significant axonal defects on their own, but combining the *ddr-1* and *ddr-2* mutations strongly enhanced axon guidance defects compared to *ddr-2* single mutants, indicating that *ddr-2* can compensate for a loss of *ddr-1*. Two different allele combinations of *ddr-1* and *ddr-2* with different genetic backgrounds showed this enhancement, suggesting that the effect is not due to unrelated background mutations. Taken together, our results demonstrate that both discoidin domain receptors of *C. elegans* have a role in axon

guidance and act in combination to guide axons along major longitudinal tracts.

The most penetrant defects in discoidin domain receptor mutants were found in the left VNC pioneer PVPR and its followers PVQL and HSNL. The PVPR axon establishes the left VNC tract and is closely followed by the PVQL axon (Durbin, 1987). PVQL is unable to pioneer the left VNC axon tract on its own and extends in the right tract in the absence of PVPR (Durbin, 1987). Post-embryonically, HSNL uses the pre-laid track formed by the axons of PVPR and PVQL as substrate (Garriga et al., 1993). We found that in almost all animals with pioneer defects axons of PVQL and HSNL followed the misguided pioneer, suggesting that defects in follower axons are secondary consequences of pioneer defects in discoidin domain receptor mutants. Consistent with this hypothesis, we found that DDR-2 expression is required in the PVPR pioneer neuron for the proper guidance of the axons of both the PVPR pioneer and the PVPL follower neurons. The navigation of the AVKR follower axon in the left VNC tract was undisturbed in *ddr-2* single and *ddr-1 ddr-2* double mutants. This was unexpected, as AVKR, like PVQL and HSNL, is thought to depend on the axon of PVPR. In a previous study no axons were found in the left VNC tract after elimination of the PVPR pioneer neuron (Durbin, 1987). However, evidence for differences between PVQL and AVKR in their dependence on the PVPR pioneer axon have also been reported in *fmi-1*/*flamingo* mutants (Steimel et al., 2010). *fmi-1* mutant animals showed penetrant guidance defects in the PVPR pioneer but only minor defects in AVKR, suggesting that the AVKR axon can navigate correctly even if the pioneer is misguided. It is possible that the PVPR pioneer is required for AVKR axon outgrowth or extension rather than navigation. Thus, the absence of the AVKR axon in the left VNC fascicle after elimination of the pioneer might simply be due to the failure of AVKR to extend its axon into the left VNC tract.

Discoidin domain receptors genetically interact with NID-1/Nidogen and FMI-1/Flamingo, but not the collagen CLE-1

Since discoidin domain receptors have been established as collagen receptors in mammals, basement membrane collagens are promising candidates as ligands. In zebrafish the basement membrane collagen type IV (Col4a5), was found to be important for proper tectal layer recognition of axons from retinal ganglion cells (Xiao and Baier, 2007). Laminar targeting by type IV collagen was independent of integrin function and achieved indirectly through anchoring heparan sulfate proteoglycans (HSPGs) and potentially other guidance cues embedded in the basement membrane, which are then used by the retinal ganglion axons for proper tectal layer targeting (Xiao and Baier, 2007). These data indicate that basement membrane collagens can affect, perhaps indirectly and in combination with other factors in the extracellular matrix, axon guidance decisions. Discoidin domain receptors have not been tested for a role in tectal layer targeting, thus a direct role for collagen IV in laminar targeting through discoidin domain receptor signaling cannot be ruled out. In *C. elegans*, the number of collagens is greatly expanded with about 160 genes compared to less than 30 in vertebrates (Kadler et al., 2007; Page and Johnstone, 2007). The vast majority of the *C. elegans* collagens are components of the cuticle (Page and Johnstone, 2007). So far, only three collagens have been associated with the basement membrane in *C. elegans*: the type IV collagens EMB-9 and LET-2 (Graham et al., 1997) and the type XV/XVIII collagen homolog CLE-1 (Ackley et al., 2001). Type IV collagens EMB-9 and LET-2 are essential structural components of the basement membrane and *emb-9* and *let-2* mutant embryos arrest at the 2-fold stage before axons start to grow out (Guo et al., 1991; Gupta et al., 1997; Sibley et al., 1994). Type XV/XVIII collagen CLE-1 strongly accumulates at the DNC and VNC and is the only collagen in *C. elegans* affecting axon guidance described so far (Ackley et al., 2001). *cle-1* mutant animals did not show defects in any of the VNC axons affected in *ddr-2* mutants, suggesting that CLE-1 does not act as ligand for discoidin domain receptors in this process. Whether other collagens interact with discoidin domain receptors in axon guidance along longitudinal tracts remains to be seen.

UNC-6/Netrin and NID-1/Nidogen are other basement membrane components with documented axon navigation defects. Mutants in both genes show axon guidance defects in the PVPR pioneer (Hutter, 2003; Kim and Wadsworth, 2000; Wadsworth et al., 1996). We found that *nid-1*/*nidogen* interacts genetically with *ddr-1* and *ddr-2*, while *unc-6*/*netrin* functions in a parallel pathway. NID-1/Nidogen is expressed in most basement membranes of *C. elegans* but accumulates on the sublateral tracts and at the muscle edges flanking the ventral and dorsal nerve cord (Kang and Kramer, 2000). Our data suggests that *nid-1*/*nidogen* and *ddr-1* and *ddr-2* act in the same genetic pathway. We speculate that the genetic interaction is indirect and does not reflect a direct physical interaction between NID-1/Nidogen and the two discoidin domain receptor proteins. Instead our results might reflect an interaction between discoidin domain receptors and collagen, which in turn interacts with NID-1/Nidogen. Mammalian Nidogen-1 and Nidogen-2 bind various basement membrane components including type IV collagen (Aumailley et al., 1993; Kohfeldt et al., 1998). Nidogen-2 was also found to bind to the ectodomain of type XIII collagen (Tu et al., 2002). Type XIII collagen is a transmembrane collagen which is strongly expressed in the developing central and peripheral nervous system of mice including the spinal cord and is able to induce neurite outgrowth (Sund et al., 2001). No clear ortholog of type XIII collagen has been identified in *C. elegans*. The GExplore protein domain search interface (Hutter et al., 2009) predicts ten transmembrane collagens in the *C. elegans* genome, all of which are uncharacterized to date.

We also tested whether discoidin domain receptors interact with other receptors known to affect PVPR guidance. Mutants in *lin-17*/*frizzled* and the non-classical cadherin *fmi-1*/*flamingo* show strong axon guidance defects in the PVPR pioneer (Steimel et al., 2010). We found that *fmi-1*/*flamingo* interacts genetically with *ddr-1* and *ddr-2* in PVPR pioneer guidance whereas *lin-17*/*frizzled* acts in a parallel pathway. *fmi-1*/*flamingo* is required cell-autonomously in PVPR for proper pioneer axon navigation (Steimel et al., 2010). FMI-1 function in the PVPR pioneer is dependent on the intracellular domain, but only partially dependent on various extracellular domains, suggesting that FMI-1 might act together with another receptor (Steimel et al., 2010). It is possible that FMI-1/Flamingo forms a receptor complex with DDR-1 and DDR-2 in PVPR to guide the PVPR pioneer axon. Further experiments will be needed to determine whether FMI-1 and the discoidin domain receptors interact physically.

In summary, our study identified a novel role for discoidin domain receptors in axon guidance during the development of the *C. elegans* nervous system. The two discoidin domain receptors of *C. elegans* work in combination to guide axons along major longitudinal tracts. We found that DDR-2 is required cell-autonomously in the PVPR neuron for the guidance of the PVPR pioneer axon, which establishes the left ventral nerve cord tract and serves as substrate for later outgrowing follower axons. Our genetic interaction studies suggest DDR-mediated pioneer guidance involves the basement membrane component NID-1/Nidogen and might require interaction with the receptor FMI-1/Flamingo.

Materials and methods

Strains and transgenes

The following alleles were used for phenotypic characterization: *ddr-1(ok874) X; ddr-1(tm382) X; ddr-2(ok574) X; ddr-2(tm797) X; lin-17(n671) I; fmi-1(rh308) V; nid-1(cg119) V; unc-6(ev400) X; cle-1(cg120) I.*

The following GFP reporter strains were used in this study: *hdIs26[odr-2::CFP, sra-6::DsRed2] rhIs4[glr-1::GFP, dpy-20(+)] III;: hdIs29[odr-2::CFP, sra-6::DsRed2] V; zdIs13[tph-1::GFP] IV; bwIs2[flp-1::GFP, rol-6(su1006)]; hdIs22[unc- 129::CFP, unc-47::DsRed2] V; evIs111[rgef-1::GFP] V.*

Strains were maintained at 20 °C under standard conditions (Brenner, 1974).

Expression constructs

The transcriptional reporter constructs *ddr-1p*::*GFP* and *ddr-2p*::*GFP* were generated by PCR amplification of the *ddr-1* and *ddr-2* upstream region (10.4 kb and 8.5 kb respectively) and cloning them into the promoterless GFP vector pPD95.75 (Fire vector kit). To generate the translational reporter constructs DDR-1::GFP and DDR-2::GFP the full sequence of *ddr-1* and *ddr-2* and 3 kb upstream sequence were cloned into pPD95.75 thus fusing GFP to the C-terminus of DDR-1 or DDR-2. All plasmid and primer sequences are available upon request. Transgenic animals were generated by injecting the reporter plasmid and a *pha-1* rescuing plasmid into *pha-1*(*e2123ts*) III animals as described (Granato et al., 1994; Mello et al., 1991).

Microscopy

Animals of a growing population were scored using a 40x objective on a Zeiss Axioscope. Images were acquired on a Zeiss Axioplan II microscope (Carl-Zeiss AG, Germany) connected to a Quorum WaveFX spinning disc system (Quorum Technologies, Canada). Stacks of confocal images with 0.2 to 0.5 μm distance between focal planes were recorded. Image acquisition and analysis was carried out using Volocity software (Perkin-Elmer, Waltham, MA). Images in the figures are maximum intensity projections of all focal planes unless otherwise mentioned. Figures were assembled using Adobe Creative Suite CS5.1 (Adobe, San Jose, CA, USA).

Analysis of neuronal defects

Axonal trajectories were examined in late-stage larvae and adult animals expressing fluorescent markers in the corresponding neurons. Cell body positions of BDU, SDQ, AVM, ALM, HSN and PVM neurons were analyzed in adult animals using the pan-neuronal marker *evIs111*[*rgef-1*::*GFP*] V. Animals were incubated with 10 mM NaN3 in M9 buffer for 1 h and mounted on agar pads prior to analysis.

Mosaic analysis

Transgenic animals for the mosaic analysis were created by injecting Fosmid WRM0624cD08 (containing the full *ddr-2* gene), the GFP reporter constructs *odr-2*::*GFP* and *sra-6*::*GFP*, and pRF4 (containing *rol-6*(*su1006*)) into *ddr-1*(*ok874*) *ddr-2*(*ok574*) X; *hdIs29*[*odr-2*::*CFP*, *sra-6*::*DsRed2*] *V* animals. These injected DNA are combined into a single extrachromosomal array, which is occasionally lost during cell divisions in the embryo, resulting in random loss in some cell lineages and mosaic expression of the transgene. Mosaic animals were scored for axon guidance defects in PVP, PVQ and AVG; the presence or absence of the rescuing extra-chromosomal array in the corresponding neuron was determined by GFP reporter expression.

Phylogenetic analysis

ClustalW2 (http://www.ebi.ac.uk/Tools/msa/clustalw2/) was used to align full gene sequences of discoidin domain receptors from *Homo sapiens sapiens*, *Mus musculus*, *Drosophila melanogaster* and *Caenorhabditis elegans*. The multiple sequence alignment file was subsequently used to generate a phylogenetic tree file (http://www.ebi.ac.uk/Tools/phylogeny/clustalw2_phylogeny/). The unrooted phylogenetic tree diagram was plotted from the phylogenetic tree file with fdrawtree (http://www.ebi.ac.uk/soaplab/).

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